Stability of mephedrone and five of its phase I metabolites in human whole blood

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1 Abstract

Mephedrone is a new psychoactive substance known to be unstable in biological matrices stored at room temperature or refrigerated. While the instability of mephedrone has been investigated before, there is currently no data regarding the stability of mephedrone metabolites. In this study, a liquid chromatography tandem mass spectrometry method for the simultaneous quantification of mephedrone and five of its phase I metabolites (dihydro-mephedrone, nor-mephedrone, hydroxytolyl-mephedrone, 4-carboxy-mephedrone and dihydro-nor-mephedrone) in human whole blood has been developed and validated. Samples were extracted by a mixed mode solid phase extraction and analyzed on a pentafluorophenylpropyl column. The method was successfully validated for selectivity, linearity (0.2-2 to 10-100 ng/mL), limits of detection (50-500 pg/mL) and quantification (200-2000 pg/mL), precision (0.924-8.27%), accuracy (86.6-115%), carryover, recovery (32.5-88.3%) and matrix effects (71.0-108%). Analyte stability in human whole blood preserved with sodium fluoride/potassium oxalate was assessed at +4°C and -20°C after 24 h, 48 h, 4 days and 10 days of storage. Instability was observed in samples stored at +4°C: nor-mephedrone and 4-carboxy-mephedrone lost 40.2 ± 6.7% and 48.1 ± 4.8%, respectively, of their initial concentration at low concentration level and 33.8 ± 4.2% and 44.6 ± 6.5%, respectively, at high concentration level after 10 days. All analytes were more stable at -20°C where the highest loss of 22.6 ± 6.9% was observed for 4-carboxy-mephedrone after 10 days. This is the first time stability of mephedrone metabolites in human whole blood has been assessed, indicating -20°C to be the recommended storage condition for all analytes in clinical settings.

2 Introduction

Mephedrone (4-methylmethcathinone) is a synthetic cathinone derivative which has a similar chemical structure and desired/adverse effects to other stimulant recreational drugs such as amphetamine [1,2]. Mephedrone was first introduced to the United Kingdom (UK) recreational drug market in 2007/8 and over the last few years it has established itself as a widely used new psychoactive substance, responsible for significant morbidity and mortality [2,3].

Despite being classified in April 2010 as a Class B drug under the UK Misuse of Drugs Act of 1971 there is some evidence suggesting that mephedrone use in the UK and in particular in London remains popular [4]. Mephedrone was detected in 1.0% (n=34) of the death cases analyzed by the Toxicology Unit at Imperial College London in 2014 and this number increased to 1.5% (n=22) in 2015 [4]. There is also evidence describing an increasing problem with people injecting mephedrone which leads to higher rates of hepatitis C, HIV and acute toxicity [5].

Studies investigating the metabolism of mephedrone have been performed *in vitro* [6,7] and *in vivo*, both in animal (rat) models [8] and in humans [9]. The main phase I metabolic pathways include N-demethylation of the secondary amine to yield nor-mephedrone (NOR), reduction of the ketone moiety to the hydroxyl group to produce dihydro-mephedrone (DHM) and oxidation of the tolyl moiety, leading to the formation of hydroxytolyl-mephedrone (HYDROXY) and 4-carboxy-mephedrone (4-CARBOXY). A simultaneous reduction of the ketone moiety and N-demethylation of the secondary amine produces dihydro-nor-mephedrone (DHNM). Hepatic CYP2D6 was found to be the main enzyme responsible for the metabolism of mephedrone in humans, with only a negligible contribution from other CYP enzymes [6].

[FIGURE 1. Mephedrone and five of its phase I metabolites]

The stability of mephedrone has previously been investigated in human whole blood containing different preservatives and stored under different conditions [10–13]. Mephedrone has been reported to be most stable at -20°C when preserved with acidic preservatives (NaF/KOx and NaF/citrate buffer). The underlying cause of its instability in biological matrices is unknown but the previous study looking at mephedrone degradation in alkaline solution suggests the involvement of oxidants such as dissolved oxygen [14].

In this study, we investigated the stability of mephedrone and five of its phase I metabolites (Figure 1) in human whole blood fortified with NaF/KOx as a preservative and anti-coagulant, respectively. A systematic stability study of the main mephedrone metabolites in whole blood has not been reported previously and therefore, this is an important investigation relevant to both clinical and forensic toxicologists.

3 Materials and methods

3.1 Reagents

Mephedrone hydrochloride (MEPH), dihydro-mephedrone hydrochloride (DHM), mephedrone-d_3 hydrochloride (MEPH-d_3), dihydro-mephedrone-d_3 hydrochloride (DHM-d_3), 4-(2-aminoethyl) benzoic acid hydrochloride (AEBA) and sodium borohydride were purchased from Sigma-Aldrich (Dorset, UK). Nor-mephedrone hydrochloride (NOR) was purchased from Chiron (Trondheim, Norway). Hydroxytolyl-mephedrone hydrochloride (HYDROXY), 4-carboxy-mephedrone hydrochloride (4-CARBOXY) as well as nor-mephedrone hydrochloride used for the in-house synthesis of dihydro-nor-mephedrone (DHNM) were purchased from LGC Standards (Bury, UK). MEPH, MEPH-d_3, DHM, DHM-d_3 were purchased as certified reference materials. All reference standards were analyzed in-house to verify their chemical structure.

All solvents were HPLC grade unless stated otherwise. Methanol (MeOH), isopropyl alcohol (IPA), dichloromethane (DCM), acetonitrile (LC-MS grade for the preparation of the mobile phase and HPLC grade for other uses), formic acid, acetic acid, sodium phosphate monobase, sodium phosphate diabase and ammonium hydroxide (0.88, 35%) were purchased from Fisher Scientific (Loughborough, UK). Ultrapure water (18 MΩcm) was prepared on an ELGA Purelab Maxima HPLC water purification system (High Wycombe, UK). Xtrackt® DAU High Flow (150 mg, 3 mL) cartridges were purchased from Chromatography Direct (Runcorn, UK).

Drug-free whole blood (5 mL) was collected by trained phlebotomists into vacutainers containing NaF/KOx (0.25% w/v/0.1% w/v). Ethical approval for the collection of drug-free matrix was granted by the Research Ethics Committee at King's College London (HR-16/17-4237).

3.2 Synthesis of dihydro-nor-mephedrone (DHNM)

Ten mg of NOR (61.3 µmol) was reduced to DHNM following a method described elsewhere [15]. Synthesized product was stored at -40°C and a small amount was characterized using high resolution mass spectrometry to determine its accurate mass (ThermoFisher Scientific Q-Exactive operated in positive electrospray ionization mode). Nuclear magnetic resonance (NMR) was also performed on a Bruker Avance DRX 400 MHz instrument.

3.3 Working solutions

Working solutions used for the preparation of the calibration curve were made in MeOH:water (50:50 v/v) at 4, 8, 16, 20, 100, 160, 200 ng/mL for MEPH, DHM, NOR, DHNM; 4, 10, 20, 100, 200, 400, 500 ng/mL for HYDROXY; and 40, 100, 200, 500, 1000, 1600, 2000 ng/mL for 4-CARBOXY. Working solution used for the preparation of the quality control samples at low, medium and high level were made in MeOH:water (50:50 v/v) at 5, 20, 160 ng/mL for MEPH, DHM, NOR, DHNM; 5, 40, 400 ng/mL for HYDROXY; and 50, 400, 1600 ng/mL for 4-CARBOXY. Internal standard (IS) solution containing

MEPH-d_3, DHM-d_3 at 50 ng/mL and AEBA at 500 ng/mL was prepared in MeOH:water (50:50 v/v).

3.4 Calibration standards (STD) and quality control (QC) samples

Matrix-matched calibration standards containing MEPH, DHM, NOR, DHNM at 0, 0.2, 0.4, 0.8, 1, 5, 8 and 10 ng/mL; HYDROXY at 0, 0.2, 0.5, 1, 5, 10, 20 and 25 ng/mL; and 4-CARBOXY at 0, 2, 5, 10, 25, 50, 80 and 100 ng/mL were prepared by the addition of an appropriate volume of the working solution to whole blood. QC Low (0.250 ng/mL for MEPH, DHM, NOR, DHNM, HYDROXY; and 2.5 ng/mL for 4-CARBOXY), Medium (1 ng/mL for MEPH, DHM, NOR, DHNM; 2 ng/mL for HYDROXY; and 20 ng/mL for 4-CARBOXY) and High (8 ng/mL for MEPH, DHM, NOR, DHNM; 20 ng/mL for HYDROXY; and 80 ng/mL for 4-CARBOXY) were prepared by the addition of an appropriate volume of the working solution to whole blood. Calibration standards and QCs were prepared fresh on the day of sample analysis.

3.5 Sample analysis

One hundred µL of whole blood (NaF/KOx) was extracted using solid phase extraction (SPE). Ten µL of IS or 10 µL of MeOH:water (50:50 v/v) was added to the samples and solvent/matrix blanks, respectively. All samples were vortex mixed and 1 mL of 0.1 M phosphate buffer (pH 6.0) was added. After conditioning the SPE cartridges (mixed mode cation exchange containing C8 and benzoylsulfonate anion) with 2 mL of MeOH and 2 mL of 0.1 M phosphate buffer (pH 6.0), samples were loaded and washed with 2 mL of 0.1 M acetic $acid_{(aq)}$ followed by 2 mL of MeOH. Samples were eluted with 4 x 1 mL of DCM:IPA:ammonium hydroxide (78:20:2 v/v/v) and dried under nitrogen at 50°C. Samples were reconstituted with 100 µL of 0.1% formic acid in ACN:water (10:90 v/v).

3.6 LC-MS/MS conditions

The analysis was performed by liquid chromatography tandem mass spectrometry (LC-MS/MS) using a Waters Xevo TQ-S triple quadrupole mass spectrometer (Manchester, UK) coupled to Waters Acquity ultra performance liquid chromatograph system equipped with a CTC 2777 open architecture autosampler (Waters, UK).

Extracted samples were analyzed using electrospray ionization operated in positive ion mode. The source temperature was set at 150°C. The desolvation gas flow rate was 1000 L/h at a temperature of 500°C, capillary voltage was set to 2.22 kV, cone voltage was 45 V and source offset was 84 V. The cone gas flow rate was set to 150 L/h, the nebulizer gas flow was 7.00 bar and the collision gas flow rate was 0.25 mL/min. Mephedrone metabolites and deuterated internal standards were monitored using selected reaction monitoring (SRM) as detailed in Table 1. In order to maximize sensitivity, all analytes except for 4-CARBOXY and HYDROXY had their dehydration products chosen as target precursor ions due to significant in-source fragmentation which is commonly observed in synthetic cathinones [9,16].

Chromatographic separation was performed using a 2.1 mm x 150 mm, 1.8 µm, pentafluorophenylpropyl Selectra® column (Bristol, US) held at 60°C. The strong needle wash was 0.3% formic acid in MeOH and the weak needle wash was 0.01% formic acid in acetonitrile:water (10:90 v/v). The flow rate was 0.5 mL/min with 0.3% formic acid in water as mobile phase A and 0.3% formic acid in acetonitrile as mobile phase B. The start of the gradient was at 85% mobile phase A. Mobile phase B was then increased to 55% over 11 min and was held for 2 min. Over the next 0.5 min the gradient returned to the starting condition and the column was re-equilibrated at 85% mobile phase A for the remaining 1.5 min. The total run time was 15 min. The injection volume was 20 µL and the data was acquired using MassLynx (version 4.1) software. TargetLynx (version 4.1) was used for data processing and quantitation.

[TABLE 1]

3.7 Stability

Stability samples prepared at QC Low and QC High levels in drug-free human whole blood (NaF/KOx) were aliquoted into tubes and stored at +4°C and -20°C for 24 h, 48 h, 4 days and 10 days. At each sampling point, one tube at each QC level was removed and six aliquots extracted. Freezer and fridge temperatures were monitored and logged daily.

4 Method validation

Validation experiments determined selectivity, linearity, inter- and intra-day precision and accuracy, limit of detection (LOD) and limit of quantification (LOQ), recovery, matrix effect, carryover and stability according to the validation guidelines published by the Food and Drug Administration (FDA) [17] and recommendations published by Peters et al. [18].

4.1 Selectivity

Selectivity was assessed by analyzing 6 blank whole blood samples collected from 3 drug-free female and 3 drug-free male donors.

4.2 Linearity

Matrix-matched calibration curve was prepared by fortifying drug-free whole blood (NaF/KOx) with appropriate working solutions containing mephedrone and its metabolites. Each calibration standard was required to be within ± 20% of its target concentration and the correlation coefficient (r^2) of the line to be at least 0.99. A linear regression model with a weighting of 1/x was applied to the calibration curve.

4.3 LOD and LOQ

The LOD for each analyte was defined as the lowest concentration where all 3 ions (2 qualifier and 1 quantifier) were present with a signal-to-noise ratio equal to or greater than 3. The LOQ was defined as the lowest concentration at which analytes could be quantified with an acceptable precision and accuracy. The upper limit of quantification was determined as the

highest concentration of the calibration line, which could be determined with an acceptable accuracy and precision without saturating the instrument signal.

4.4 Precision and accuracy

Intra-day (n=6) and inter-day (n=3) precision and accuracy was determined by employing QC samples spiked at low, medium and high concentrations. Intra-day precision was calculated using 6 replicates obtained on the same day and expressed as a percentage relative standard deviation (% RSD). Accuracy was calculated by dividing the mean measured concentration at each QC level by the theoretical spiked concentration and expressed as a percentage of the theoretical spiked concentration. Inter-day precision was evaluated for each QC level run on three different days and expressed as % RSD. Values of ± 15% and ± 20% at LOQ are acceptable according to the guidelines.

4.5 Recovery and matrix effect

For recovery, a set of whole blood samples (n=6) was fortified at QC Low and QC High level and was taken through the SPE. In parallel, a set of blank whole blood samples (n=6) was extracted and fortified after the evaporation step at QC Low and QC High level. Recovery was calculated as a percentage by comparing the absolute peak areas of the samples spiked before extraction with samples spiked after extraction.

For the IS-corrected matrix effect, a set of blank whole blood samples (n=6, from 3 female and 3 male individuals) and a set of water samples (n=6) was taken through the extraction. All samples were reconstituted with a solution containing known amounts of the internal standard and analytes at QC Low and QC High levels. Matrix effect was evaluated by comparing peak area ratios in blank whole blood samples fortified after extraction with peak area ratios in water samples fortified after extraction.

4.6 Carryover

Carryover was assessed by injecting methanol blanks after the highest calibration standard.

5 Results and Discussion

5.1 DHNM synthesis

DHNM was successfully synthesized (yield: 51%). Formula $C_{10}H_{16}NO^{+}$; HRMS [$M+H^{+}$] calculated m/z 166.1226, observed 166.1227 (+0.001 ppm); observed MS/MS fragments with collision energy 20 eV were consistent with those reported in the literature [9]. ^{1}H NMR ($CDCl_3$): δ 7.22 (d, J=8.0 Hz, 2H, Ar-H), 7.16 (d, J=8.0 Hz, 2H, Ar-H), 4.50 (d, J=4.0 Hz, 1H, C<u>H</u>(OH)), 3.19 (br , 1H, C<u>H</u>(CH_3)), 2.35 (s, 3H, Ar-CH_3) and 0.98 (d, J=8.0 Hz, 3H, CH(C<u>H</u>$_3$)). ^{1}H NMR data is consistent with the literature except for the signal at 3.19 ppm being previously reported as a multiplet [15].

5.2 Method validation

Selectivity

No interferences were observed in the extracted blank matrix.

Linearity

Mean linearity of $r^2 > 0.998$ was achieved for each analyte in all 3 validation runs.

LOD and LOQ

LOD and LOQ of 50 pg/mL and 200 pg/mL, respectively, was achieved for all analytes except 4-CARBOXY for which the LOD was 500 pg/mL and the LOQ 2000 pg/mL, the order of magnitude difference observed most probably being due to reduced electrospray ionization efficiency of this amphiprotic molecule under the mobile phase conditions chosen. Table 2 shows calibration parameters for all analytes.

[TABLE 2]

Precision and accuracy

Intra-day and inter-day precision and accuracy results, summarized in Table 3**,** were found to be within the acceptable limits. The intra-day accuracy for all metabolites was within ± 15% of the target concentration and ranged from 96.7-106% for MEPH, 91.1-109% for DHM, 89.7-97.0% for NOR, 94.3-115% for HYDROXY, 97.0-114% for 4-CARBOXY and 86.6-103% for DHNM. The intra-day precision was < 7% and ranged from 1.44-4.33% for MEPH, 0.924-4.65% for DHM, 1.58-4.87% for NOR, 1.55-6.57% for HYDROXY, 1.36-5.97% for 4-CARBOXY, 1.52-5.13% for DHNM. Inter-day precision and accuracy results were acceptable over the validated range with % RSD < 8.5% and accuracy within ± 9.0% of the target concentration.

[TABLE 3]

Recovery and matrix effect

Recovery was found to be greater than 71.3% for all analytes, except for 4-CARBOXY for which recovery was 32.5 ± 6.8% at QC Low level and 41.6 ± 0.5% at QC High level. 4-CARBOXY is a zwitterionic compound which contains an acidic carboxylic acid group and a basic secondary amine group. According to Marvin (chemistry software package, version 17.16.0), the secondary amine has a pKa of 8.0 and the carboxylic acid has a pKa of 3.6, similar to the pKa values of mephedrone and benzoic acid, respectively. Secondary amines become fully protonated upon the addition of diluted acetic acid (pH 2.9) during the wash step in SPE, whereas only about 80% of the carboxylic acid group is protonated under this pH. Therefore, about 20% of the molecule exists as a neutral zwitterion with no net charge. A subsequent wash with 2 mL of MeOH to remove neutral and acidic interferents, such as free fatty acids, will also disrupt the hydrophobic interaction of this ‘net neutral’ metabolite with the C8 alkyl chains, causing a considerable proportion to be lost which might explain lower recovery for 4-CARBOXY. The other analytes are all basic and as cations will ionically interact with the benzoylsulfonate anion within the mixed-mode stationary phase during the MeOH wash, which results in higher recovery. Even though it is recommended for the

recovery to be greater than 50% [18], desired sensitivity as well as acceptable precision and accuracy were achieved for 4-CARBOXY (Table 4).

IS-corrected matrix effect values were within ± 17% at both QC Low and QC High level, except for HYDROXY at QC High which was suppressed by 29.0% (Table 4). This may be due to the lack of matching deuterated IS which is currently not commercially available. However, assay precision and accuracy for HYDROXY at QC High was within the acceptable limits (Table 3).

[TABLE 4]

Carryover

Carryover was not observed.

5.3 Stability

Stability data together with corresponding % RSD is presented in Table 5 and showed graphically in Figures 2-5. Analytes were considered unstable when they lost more than 10% of their initial concentration.

At +4°C at QC Low, DHM and DHNM were stable over the 10-day period while HYDROXY and MEPH lost 18.6 ± 5.2% and 23.4 ± 6.3%, respectively, of their initial concentration. 4-CARBOXY and NOR decreased in concentration by 48.1 ± 4.8% and 40.2 ± 6.7%, respectively, after 10 days (Figure 2). At QC High, DHM and DHNM were stable over the 10-day period while HYDROXY and MEPH lost 11.3 ± 3.2% and 14.2 ± 3.3%, respectively, of their initial concentration. 4-CARBOXY and NOR were most unstable and their concentration decreased by 44.6 ± 6.5% and 33.8 ± 4.2%, respectively, after 10 days (Figure 3).

[FIGURE 2. Analyte stability at QC Low at +4°C]

[FIGURE 3. Analyte stability at QC High at +4°C]

At -20°C at QC Low, NOR and DHNM were most stable over the 10-day period while MEPH, 4-CARBOXY, HYDROXY and DHM lost 9.9 ± 2.4%, 9.6 ± 5.3%, 11.2 ± 4.8% and 12.0 ± 4.8%, respectively, of their initial concentration (Figure 4). At QC High, 4-CARBOXY was the most unstable and decreased in concentration by 22.6 ± 6.9% after 10 days. MEPH, DHM, NOR and DHNM were stable over the 10-day period while HYDROXY lost 10.2 ± 2.2% of its initial concentration (Figure 5).

[FIGURE 4. Analyte stability at QC Low at -20°C]

[FIGURE 5. Analyte stability at QC High at -20°C]

Out of all metabolites, 4-CARBOXY was the most unstable at +4°C with significant losses observed already after 4 days (33.7 ± 6.1%) at QC Low and after 48 h (21.6 ± 4.3%) at QC High. Its stability was improved at -20°C where the highest loss of 22.6 ± 6.9% was observed after 10 days at QC High. NOR was much more stable at -20°C than 4°C where after 10 days it lost 40.2 ± 6.7% at QC Low (versus no loss at -20°C) and 33.8 ± 4.2% at QC High (versus loss of 6.6 ± 3.6% at -20°C). After 10 days HYDROXY was unstable at both storage conditions where the highest loss of 18.6% ± 5.2% was observed at QC Low at +4°C. As

opposed to other analytes which showed significant losses at +4°C after 10 days, DHM and DHNM were stable at both concentration levels when refrigerated, with the latter showing a slight increase in its concentration after 10 days at QC High. DHM and DHNM are the only two metabolites containing a hydroxyl group instead of a ketone at the β carbon which was previously reported to make ephedrines more stable than cathinones [11].

[TABLE 5]

Studies investigating the stability of mephedrone in human whole blood have been published before. Sørensen [11] investigated the stability of cathinones (including mephedrone) and related ephedrines in human whole blood spiked with analytes at 100 µg/mL and preserved with NaF/KOx or NaF/citrate buffer. Samples were stored at either +4°C or +20°C for up to 5 or 6 days. After 5 days of storage mephedrone was more stable at +4°C than +20°C. Busardò et al. [12] reported on the stability of mephedrone in ante-mortem and post-mortem blood preserved with NaF/KOx or EDTA. Whole blood samples were spiked at 1 mg/mL and stored at -20°C, +4°C or +20°C for up to 185 days. Mephedrone was shown to be most stable in ante-mortem samples at all tested storage conditions, with -20°C being the best storage temperature. This study showed that mephedrone stability is pH dependent and acidic preservatives are better suited (6.6% vs 9.4% loss after 185 days at -20°C when preserved with NaF/KOx rather than EDTA). Johnson and Botch-Jones [10] investigated the stability of four designer drugs (including mephedrone) stored at -20°C, +4°C or +22°C over 14 days. Human whole blood (preservative not stated), plasma and urine samples were spiked at 1 µg/mL. The study showed a mean 48% reduction in mephedrone concentration in whole blood kept at +4°C for 14 days. Over the same period of time mephedrone was undetected when stored at room temperature whilst there was no measurable degradation at -20°C. The most recent study looked at the stability of mephedrone and other synthetic cathinones in bovine blood fortified with NaF/KOx at 100 ng/mL (QC Low) and 1,000 ng/mL (QC High) stored at -20°C, +4°C, +20°C and +32°C. At QC Low a complete degradation of mephedrone was observed after 11 days when stored at the elevated temperature. Degradation was much slower at +4°C and -20°C where a 20% loss was observed after 55 days and 130 days, respectively [13]. These results follow the stability pattern seen in our study where mephedrone and its metabolites have been shown to be more stable at -20°C than +4°C.

For stability analysis, the QCs prepared were the same as those used in a pharmacokinetic investigation, analyzing whole blood from five healthy volunteers who had insufflated a 100 mg dose of mephedrone powder. The results from that study are being prepared for publication, but it is germane to report here that the whole blood concentrations of the metabolites in the subjects were similar to the concentrations chosen for the low, medium and high quality controls. For determination of mephedrone concentrations in the pharmacokinetic study, the need for sample dilution was anticipated and indeed required to achieve readings within the calibration range and around the mephedrone concentrations in the QCs, as well as to avoid ion overload. The focus of our stability study was on the metabolites of mephedrone, the results of which, to the best of our knowledge, are novel. Even so, our findings concerning the stability of mephedrone itself, albeit at very much

smaller concentrations compared to cases associated typically with acute toxicity, appear to be in keeping with previous stability studies using higher amounts (cited above).

6 Conclusion

A fully validated method for the simultaneous quantification of mephedrone and five of its phase I metabolites in human whole blood (NaF/KOx) has been developed. Mephedrone's stability in whole blood has been previously investigated but this is the first time stability of its metabolites has been assessed, indicating -20°C to be the recommended storage condition for all analytes.

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***Table 1**. The retention time, SRM transitions and collision energy for each ion; * denotes a quantifying transition*

Analyte	**Retention time** (min)	**Precursor ion** (*m/z*)	**Product ion** (*m/z*)	**Collision energy** (V)	**Internal standard**
MEPH	5.85	160.4	145.1*	15	MEPH-d_3
			144.1	33	
			91.1	28	
MEPH-d_3	5.85	163.4	148.4	19	
DHM	5.38	162.4	147.3*	19	DHM-d_3
			131.4	17	
			91.3	26	
DHM-d_3	5.38	165.4	150.3	18	
NOR	5.00	146.0	131.1	25	MEPH-d_3
			130.1*	25	
			119.0	15	
HYDROXY	1.98	194.1	158.1	17	DHM-d_3
			146.0*	17	
			131.1	23	
4-CARBOXY	2.06	208.0	146.0*	13	AEBA
			144.1	28	
			130.1	31	
DHNM	4.45	148.1	131.1*	13	MEPH-d_3
			116.2	23	
			91.1	25	
AEBA	1.77	166.1	149.1	10	

Table 2. *LOD, LOQ, calibration range and calibration parameters for all analytes*

Analyte	**LOD** (pg/mL)	**LOQ** (pg/mL)	**Range** (ng/mL)	**Intercept ± SD** (n=3)	**Slope ± SD** (n=3)	**r^2 ± SD** (n=3)
MEPH	50	200	0.2-10	-0.0127 ± 0.0263	3.15 ± 0.1290	0.999 ± 0.0000
DHM	50	200	0.2-10	-0.0324 ± 0.0043	2.81 ± 0.0529	0.998 ± 0.0010
NOR	50	200	0.2-10	-0.0147 ± 0.0088	1.65 ± 0.0608	0.997 ± 0.0000
HYDROXY	50	200	0.2-25	-0.000510 ± 0.0096	1.21 ± 0.1300	0.998 ± 0.0010
4-CARBOXY	500	2000	2-100	6.85 ± 5.8800	27.9 ± 23.6000	0.997 ± 0.0015
DHNM	50	200	0.2-10	-0.00381 ± 0.0073	2.10 ± 0.2000	0.998 ± 0.0006

Table 3*. Precision and accuracy at QC Low, QC Medium and QC High;* [*] *average value of 18 measurements over 3 days*

Analyte	True value (ng/mL)	**Mean** (ng/mL), **(% RSD)**, **% accuracy**			
		Day 1 n=6	Day 2 n=6	Day 3 n=6	Inter-day n=3[*]
MEPH	0.250	0.246 (4.33%) 98.2%	0.251 (1.44%) 101%	0.248 (3.45%) 99.3%	0.248 (3.24%) 99.3%
	1	1.00 (2.92%) 100%	0.967 (2.33%) 96.7%	1.00 (1.88%) 100%	0.988 (2.77%) 98.8%
	8	8.47 (1.72%) 106%	7.92 (2.40%) 99.0%	7.88 (1.48%) 98.6%	8.08 (3.84%) 101%
DHM	0.250	0.273 (2.68%) 109%	0.228 (2.80%) 91.1%	0.256 (4.65%) 102%	0.252 (8.27%) 101%
	1	1.02 (4.06%) 102%	0.992 (1.74%) 99.2%	1.04 (2.98%) 104%	1.02 (3.53%) 102%
	8	8.15 (0.924%) 102%	7.84 (2.22%) 98.0%	7.79 (4.05%) 97.3%	7.91 (3.28%) 98.9%
NOR	0.250	0.229 (2.55%) 91.6%	0.238 (2.97%) 95.1%	0.231 (4.87%) 92.5%	0.233 (3.77%) 93.1%
	1	0.933 (3.01%) 93.3%	0.928 (3.63%) 92.8%	0.932 (1.58%) 93.2%	0.931 (2.70%) 93.1%
	8	7.76 (3.87%) 97.0%	7.40 (4.56%) 92.5%	7.18 (3.63%) 89.7%	7.44 (5.03%) 93.0%
HYDROXY	0.250	0.236 (5.53%) 94.3%	0.267 (4.70%) 107%	0.253 (2.72%) 101%	0.252 (6.69%) 101%
	2	2.07 (3.49%) 103%	2.29 (3.89%) 115%	2.14 (2.37%) 107%	2.17 (5.42%) 108%
	20	21.1 (6.57%) 105%	23.0 (4.75%) 115%	20.1 (1.55%) 100%	21.4 (7.38%) 107%
4-CARBOXY	2.5	2.42 (1.36%) 97.0%	2.48 (3.93%) 99.1%	2.43 (4.26%) 97.0%	2.44 (3.48%) 97.7%
	20	20.7 (5.44%) 103%	21.9 (5.97%) 109%	22.9 (3.95%) 114%	21.8 (6.40%) 109%
	80	85.2 (5.40%)	85.3 (3.37%)	87.6 (3.60%)	86.1 (4.21%)

		107%	107%	109%	108%
DHNM	0.250	0.228 (3.46%) 91.3%	0.238 (4.55%) 95.0%	0.239 (1.52%) 95.4%	0.235 (3.78%) 93.9%
	1	0.970 (5.13%) 97.0%	0.922 (3.72%) 92.2%	0.937 (4.99%) 93.7%	0.943 (4.92%) 94.3%
	8	8.22 (4.00%) 103%	7.49 (4.01%) 93.6%	6.93 (2.21%) 86.6%	7.55 (7.97%) 94.3%

Table 4. *Analyte recovery and matrix effect at QC Low and QC High*

Analyte	**Recovery (% RSD)**, n=6		**Matrix Effect (% RSD)**, n=6	
	QC LOW	QC HIGH	QC LOW	QC HIGH
MEPH	85.2% (1.84%)	88.3% (3.17%)	101% (8.16%)	99.0% (1.12%)
DHM	83.6% (9.97%)	84.2% (2.70%)	105% (2.87%)	98.7% (0.792%)
NOR	74.3% (3.78%)	76.6% (3.31%)	89.6% (5.89%)	91.7 % (3.25%)
HYDROXY	71.3% (4.12%)	81.4% (2.62%)	83.8% (2.58%)	71.0% (6.00%)
4-CARBOXY	32.5% (6.79%)	41.6% (0.522%)	103% (6.49%)	108% (5.99%)
DHNM	78.6% (5.70%)	79.0% (5.41%)	93.0% (4.66%)	87.4% (4.94%)

Table 5. *QC Low and QC High concentrations ± % RSD (% loss/gain) for each analyte after 24 h, 48 h, 4 days and 10 days of storage at +4°C and -20°C*

Analyte	QC level, conc. (ng/mL)	24 h		48 h		4 Days		10 Days	
		+4°C	**-20°C**	**+4°C**	**-20°C**	**+4°C**	**-20°C**	**+4°C**	**-20°C**
MEPH	QC Low, 0.250	0.239 ± 1.3% (-4.4%)	0.246 ± 5.5% (-1.8%)	0.238 ± 5.8% (-4.9%)	0.259 ± 0.31% (+4.0%)	0.238 ± 4.3% (-4.9%)	0.260 ± 5.80% (+4.0%)	0.192 ± 6.3% (-23.4%)	0.225 ± 2.4% (-9.9%)
	QC High, 8	7.53 ± 2.6% (-5.8%)	8.39 ± 4.1% (+5.0%)	7.79 ± 2.2% (-2.6%)	8.63 ± 3.5% (+8.0%)	7.74 ± 3.8% (-3.3%)	8.25 ± 5.1% (+3.0%)	6.86 ± 3.3% (-14.2%)	7.88 ± 2.0% (-1.5%)
DHM	QC Low, 0.250	0.250 ± 2.1% (-0.1%)	0.243 ± 4.0% (-2.8%)	0.262 ± 7.3% (+5.0%)	0.243 ± 2.7% (-2.8%)	0.256 ± 1.1% (+2.0%)	0.240 ± 3.9% (-4.0%)	0.237 ± 3.7% (-5.0%)	0.220 ± 4.8% (-12.0%)
	QC High, 8	7.52 ± 2.9% (-6.0%)	8.36 ± 4.8% (+5.0%)	7.82 ± 0.86% (-2.2%)	8.52 ± 3.9% (+7.0%)	8.17 ± 1.9% (+2.0%)	8.24 ± 5.1% (+3.0%)	8.10 ± 0.62% (1.0%)	7.79 ± 2.0% (-2.6%)
NOR	QC Low, 0.250	0.245 ± 4.0% (-1.8%)	0.248 ± 3.2% (-0.8%)	0.252 ± 5.7% (+1.0%)	0.246 ± 7.0% (-1.4%)	0.225 ± 4.8% (-10.0%)	0.255 ± 7.2% (+2.0%)	0.149 ± 6.7% (-40.2%)	0.258 ± 7.5% (+3.0%)
	QC High, 8	7.41 ± 3.1% (-7.4%)	8.36 ± 4.1% (+4.0%)	7.84 ± 2.5% (-2.0%)	8.83 ± 4.9% (+10.0%)	6.99 ± 4.0% (-12.6%)	8.04 ± 3.4% (0.0%)	5.30 ± 4.2% (-33.8%)	7.48 ± 3.6% (-6.6%)
HYDROXY	QC Low, 0.250	0.242 ± 5.6% (-3.3%)	0.235 ± 3.4% (-6.0%)	0.256 ± 10.0% (+2.4%)	0.239 ± 4.2% (-4.4%)	0.234 ± 8.8% (-6.4%)	0.221 ± 4.7% (-11.4%)	0.203 ± 5.2% (-18.6%)	0.222 ± 4.8% (-11.2%)
	QC High, 20	18.1 ± 0.93% (-9.7%)	18.8 ± 5.3% (-5.9%)	18.2 ± 4.9% (-8.9%)	19.2 ± 5.3% (-4.0%)	19.3 ± 3.6% (-3.6%)	19.3 ± 4.9% (-3.5%)	17.7 ± 3.2% (-11.3%)	18.0 ± 2.2% (-10.2%)
4-CARBOXY	QC Low, 2.5	2.38 ± 5.0% (-4.8%)	2.49 ± 5.8% (-0.5%)	2.54 ± 5.1% (+2.0%)	2.56 ± 6.9% (+2.0%)	1.66 ± 6.1% (-33.7%)	2.61 ± 6.5% (+4.0%)	1.30 ± 4.8% (-48.1%)	2.26 ± 5.3% (-9.6%)
	QC High, 80	68.6 ± 5.4% (-14.2%)	78.6 ± 2.2% (-1.7%)	62.7 ± 4.3% (-21.6%)	87.5 ± 10.8% (+9.0%)	63.9 ± 4.6% (-20.2%)	81.8 ± 8.3% (+2.0%)	44.3 ± 6.5% (-44.6%)	61.9 ± 6.9% (-22.6%)
DHNM	QC Low,	0.255 ±	0.253 ±	0.245 ±	0.251 ±	0.247 ±	0.251 ±	0.236 ±	0.254 ±

	0.250	1.9% (+2.0%)	0.71% (+1.0%)	7.9% (-1.9%)	6.5% (0.0%)	5.2% (-1.4%)	3.9% (0.0%)	3.7% (-5.7%)	4.9% (+2.0%)
	QC High, 8	7.33 ± 1.8% (-8.4%)	8.51 ± 0.72% (+6.0%)	7.90 ± 2.3% (-1.3%)	8.62 ± 1.5% (+8.0%)	8.06 ± 3.7% (+1.0%)	8.18 ± 2.9% (+2.0%)	8.57 ± 4.8% (+7.0%)	7.39 ± 3.3% (-7.6%)

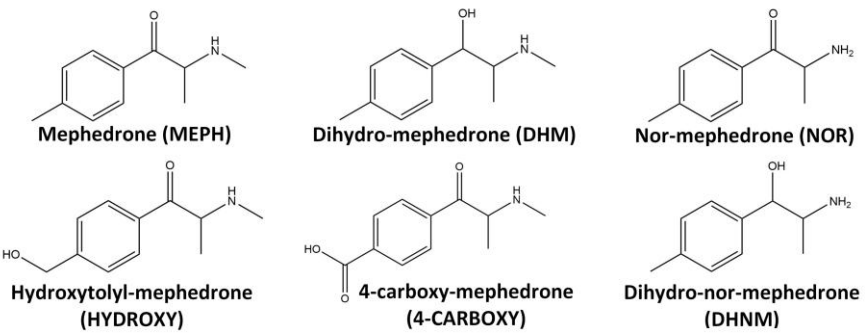

Figure 1. Mephedrone and five of its phase I metabolites

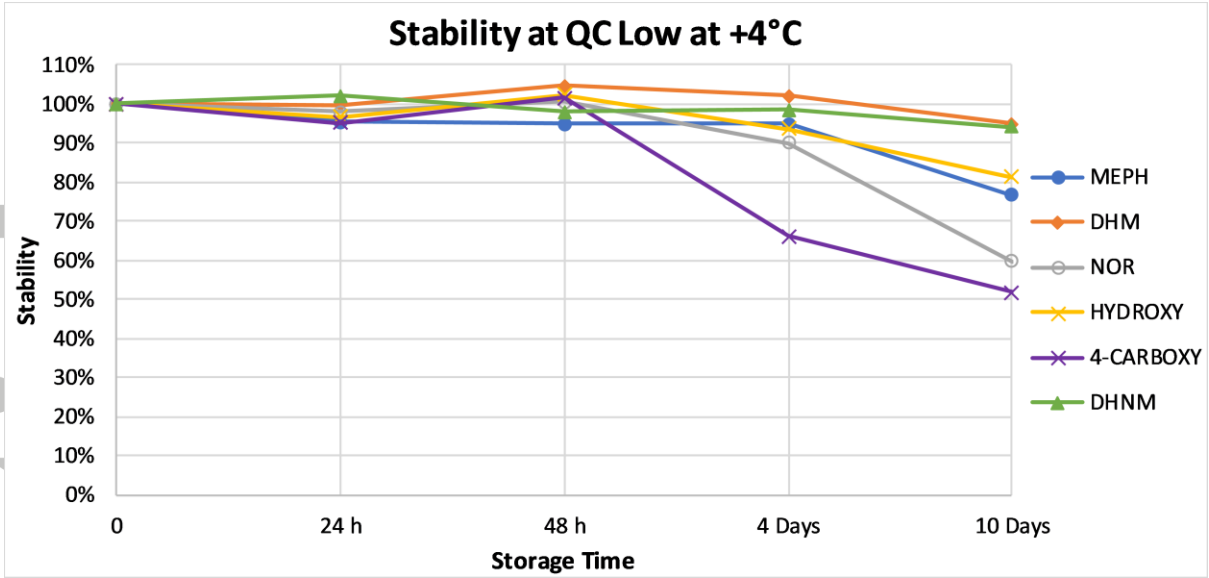

Figure 2. Analyte stability at QC Low at +4°C

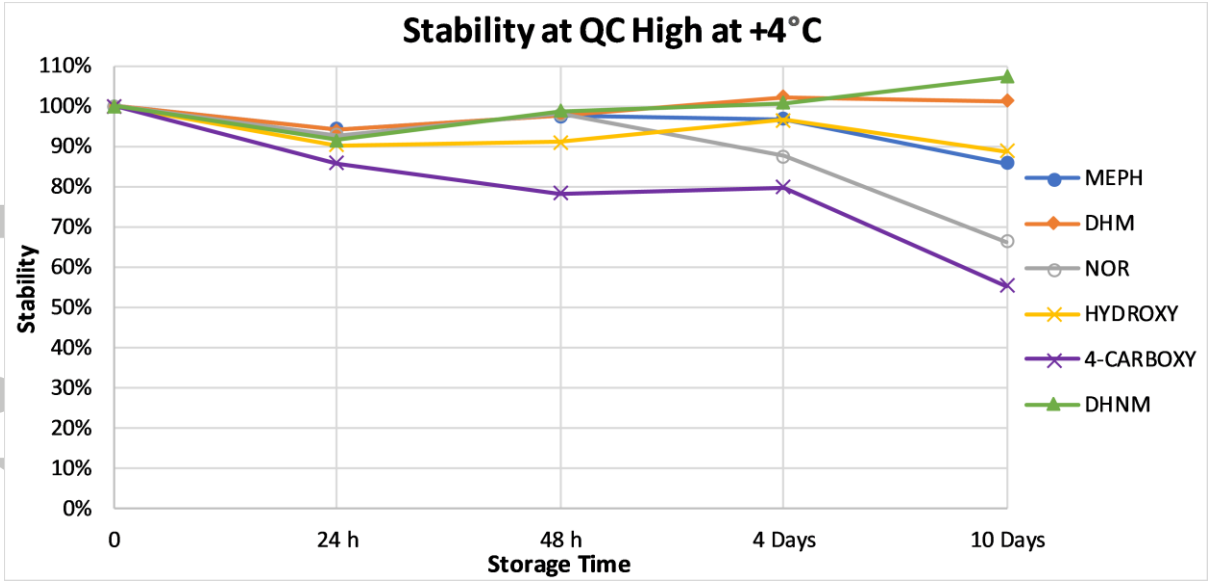

Figure 3. Analyte stability at QC High at +4°C

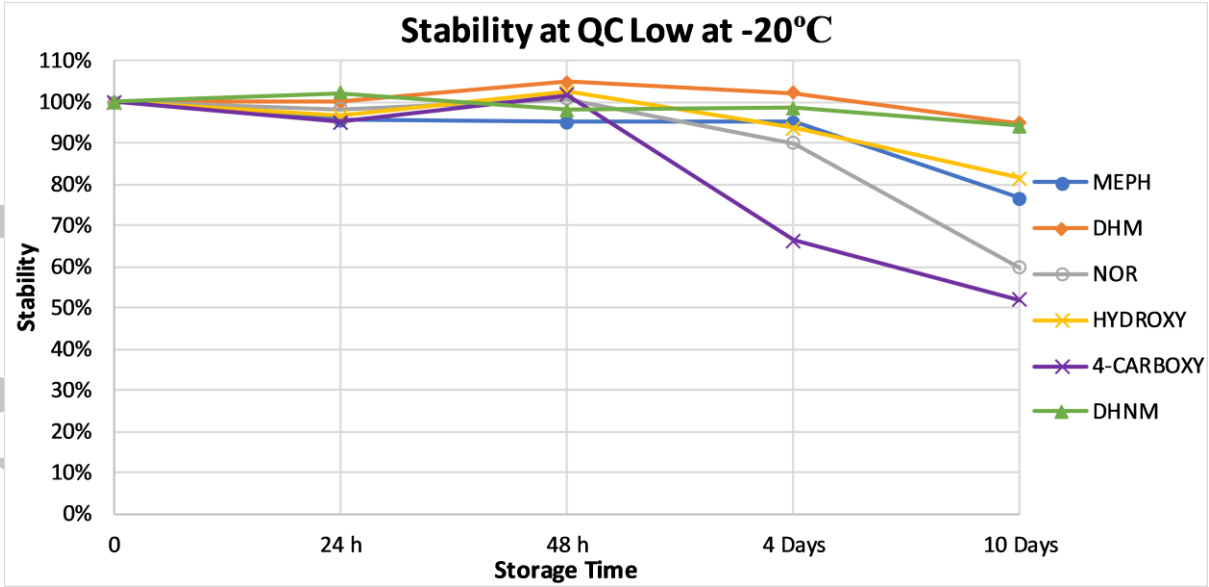

Figure 4. Analyte stability at QC Low at -20°C

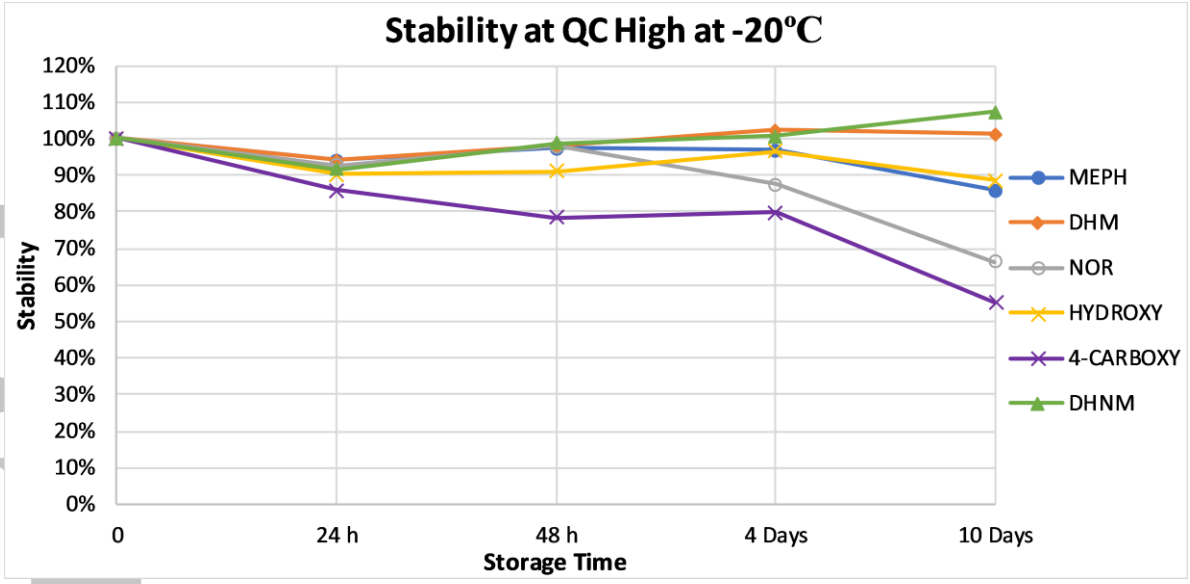

Figure 5. Analyte stability at QC High at -20°C